

# Translational Tissue Modeling Laboratory

## **Generation of LWRN Conditioned Media**

Protocol based on Miyoshi et al, 2013 PMC3969856

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## **Description:**

This protocol describes how to generate media containing mouse Wnt-3A, R-spondin 3, and Noggin used for generating and maintaining mouse and human enteroid/colonoid cultures.

### **Equipment & Materials:**

- Tissue Culture Hood
- L-WRN Cells (<u>ATCC CRL-3276</u>)
  - Growth Media: Advanced DMEM/F12 (Gibco, 12634-028) + 20% FBS (Gibco, 26140-079; ≤10EU/mL) +
    1% GlutaMAX (Gibco, 35050-061) + 1% Pen/Strep (final 100 U/mL; Gibco, 15140-122)
  - Selection: Geneticin/G418 & Hygromycin (both 0.5 mg/mL)
- 15 cm Petri Dishes
- Greiner Bio-One CELLdisc™ Multilayer Cell Culture Surface (Fisher 07001057) for large-scale productions
- 500 mL Vacuum Filtration Units (ThermoFisher 569-002 Rapid Flow 0.2μM PES membrane 90mm filter)
- 250-500 mL Sterile PETG Media Bottles (ThermoFisher FBMB250)
- 50 mL Conicals
- 1.5 mL Centrifuge Tubes

#### Methods:

- 1. Thaw LWRN cells in 30mL Growth Media and culture the cells in one 15cm Petri Dish.
- 2. The next day, depending on cell recovery, change media with <u>dual selection</u> and grow to about 80-90% confluency (usually ~ five days) without media change.
- 3. Discard medium, rinse flask with warm DPBS to remove residual selection antibiotic.
- 4. Split cells into 10 new plates without antibiotic selection.
- 5. Add 25 mL of Growth Media per plate.
- 6. Once cells are about 80% confluent (usually ~2 days), collect and replace media on each plate **every day** for 10-12 consecutive days.
- 7. Save 500  $\mu$ L of media into a centrifuge tube from each day for testing activity using the TOPflash Wnt Reporter and mycoplasma testing. Assay and store at 4°C.
- 8. Spin at 500xg for 10 minutes, or, allow debris to sediment overnight at 4°C. The following morning, filter sterilize and aliquot into 25 or 50 mL volumes and store aliquots at -80°C. (e.g., filtering, aliquoting, and freezing media from day 1 on Day 2).



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## **LWRN QC**

- 1. Limit freeze-thaws to 2 times. Once thawed, the LWRN is functional for 14 days when stored at 4C (VanDussen et al, 2019 PMC6579736)
- 2. L-WRN conditioned media contains approximately 45 ng/mL Wnt-3A, 25ng/mL R-spondin 3, and 25 ng/mL noggin (Speer, JE... Nancy L. Allbritton. Evaluation of human primary intestinal monolayers for drug metabolizing capabilities. J Biol Eng 13, 82 (2019) doi:10.1186/s13036-019-0212-1)
- 3. The TTML also performs a Wnt assay and uses this as a guide. The below 7 day functional test on each collection day ultimately determines usage.

### **Functional QC**

- 1. We test one of our most sensitive colonoid lines, which is Colon-87.
- 2. Each LWRN collection day is used to set up a well of colonoids (24-well plate; four 10uL drops each well). Medium is also added to the matrigel as described in our methods.
- 3. Two positive controls: IntestiCult-human (StemCell Technologies; 06010) and a good performing mix from the previous batch.
- 4. Negative control: usually day-1 or the late days, 10-12, will serve as an internal low performer.
- 5. Medium is refreshed every day.
- 6. At the end of the 7 days we document by imaging and also blindly count dead/differentiated structures for each day.
- 7. Based on results, we combine individual high and low proliferation days and retest. Ideally, we test and refreeze mixes from 3-4 days in working volumes of 50 mLs.
  - a. See this <u>QC example</u>. If the medium produces a majority of structures that are grey/black, we will likely not use it at all (Day 1/2 example), unless we have human fetal or mouse colonoids in culture which requires less Wnt.
  - b. If some shiny structures are evident (Day 9/10 or 11/12 example) then this suggests new growth and we will attempt to salvage that day by combining it with medium that had very minimal death. In this example we may combine Day 5/6, 7/8, and 11/12. The first time we make this particular combination, with each individual batch, we will test alongside a working combination. We define a working combination as one that produces approximately 90% clear shiny cystic structures in Colon-87. Most of the time we hit the mark, but occasionally we are surprised and have to adjust, that is, we have to add another high-performing day to the mix.
  - c. So the goal is not maximal activity for colonoid maintenance. For certain applications we do indeed push towards maximal activity, such as preparation for 2D monolayers.